Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution

(histocompatibility/transplantation/hematopoiesis/stem cells/blood banking)

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ABSTRACT   Clinical evidence of hematopoietic restoration with placental/umbilical cord blood (PCB) grafts indicates that PCB can be a useful source of hematopoietic stem cells for routine bone marrow reconstitution. In the unrelated setting, human leukocyte antigen (HLA)-matched donors must be obtained for candidate patients and, hence, large panels of frozen HLA-typed PCB units must be established. The large volume of unprocessed units, consisting mostly of red blood cells, plasma, and cryopreservation medium, poses a serious difficulty in this effort because storage space in liquid nitrogen is limited and costly. We report here that almost all the hematopoietic colony-forming cells present in PCB units can be recovered in a uniform volume of 20 ml by using rouleaux formation induced by hydroxyethyl starch and centrifugation to reduce the bulk of erythrocytes and plasma and, thus, concentrate leukocytes. This method multiplies the number of units that can be stored in the same freezer space as much as 10-fold depending on the format of the storage system. We have also investigated the proportion of functional stem/progenitor cells initially present that are actually available to the recipient when thawed cryopreserved PCB units are infused. Progenitor cell viability is measurably decreased when thawed cells, still suspended in hypertonic cryopreservative solutions, are rapidly mixed with large volumes of isotonic solutions or plasma. The osmotic damage inflicted by the severe solute concentration gradient, however, can be averted by a simple 2-fold dilution after thawing, providing almost total recovery of viable hematopoietic progenitor cells.

It is now known that the concentrations of hematopoietic progenitor cells in placental/umbilical cord blood (PCB) collections are similar (1–3) to those in collections of bone marrow for transplantation. The long-term hematopoietic reconstitution of a Fanconi anemia patient with PCB from an unaffected sibling demonstrated that PCB collections contain sufficient stem cells for allogeneic bone marrow restoration (4). Since the initial case, several dozen patients with genetic diseases and hematological malignancies (5) have been transplanted with allogeneic PCB from siblings, both human leukocyte antigen (HLA) identical and partially mismatched (6–8), and from HLA-matched, unrelated donors (8). A remarkable attribute of these transplants has been their relative freedom from graft-versus-host disease (J. Kurtzberg, E. Gluckman, J. E. Wagner, and D. Emanuel, personal communications), which is a leading cause of morbidity and mortality in bone-marrow transplant (BMT) recipients (reviewed in ref. 9). Other advantages of PCB as donor tissue for unrelated bone marrow replacement are the lower frequency of some infections and the promptness with which PCB can be made available to patients (reviewed in ref. 10). Wider utilization of

this resource, ignored until recently, may be anticipated given the success of its clinical application, its abundance, and its ability to be recovered with comparative ease and without risk to either the neonatal donors or their mothers.

The New York Blood Center's Placental Blood Project (11) began collecting PCB units in February 1993 in a study of the clinical usefulness of this source of stem cells. As of February 26, 1995, it had cryopreserved 3814 PCB units and provided transplants to 24 unrelated recipients [the first two such transplants were reported in 1993 (8)]. Of the 24 patients grafted before February 26, 1995, 12 engrafted and were alive and well as of March 30, 1995; 3 with demonstrated engraftment died of fungal infections before day 27; 3 had leukemic relapses (one after documented engraftment); 2 transplants failed, but the patients are alive (one had autologous hematopoietic recovery and the second has been subsequently retransplanted); and 4 recipients died within 2 weeks of the transplant and could not be evaluated for engraftment. The 16 cases who engrafted had nucleated blood cells and/or bone marrow of donor type by serologic and PCR criteria or by restriction fragment length polymorphism. None of the patients developed clinically severe graft-versus-host disease within follow-up periods of 2–18 months. Two additional unrelated PCB grafts have been performed that also engrafted and are clinically well after several months; one by E. Gluckman (personal communication) and the other from the Cord Blood Bank of Milan (personal communication). Thus, like their related counterparts, unrelated PCB transplants generally engraft and produce little or no severe graft-versus-host disease.

We now address two issues that are essential for the full development of unrelated-donor PCB transplantation as a practical alternative for clinical use. The first problem is that stockpiling ("banking") a sufficiently large number of cryoprotected whole PCB units requires vast amounts of costly storage space in liquid nitrogen (LN). To establish an adequate panel, therefore, the hematopoietic cells of PCB units need to be concentrated into units of much smaller volume. However, Broxmeyer et al. (1) found unacceptably high progenitor cell losses when using techniques for red blood cell separation such as simple centrifugation, lysis with ammonium chloride, differential settling in viscous media, and filtration through density gradients. Other investigators reported better recoveries (12–14), but their procedures required transferring the blood from collection bags to other vessels, thereby exposing the blood to the risk of bacterial and fungal contamination and increasing the possibility of identification errors. These manipulations are also time and labor intensive and do not fit easily in the routine demanded by medium- and large-scale PCB processing and storage.

Abbreviations: PCB, placental/umbilical cord blood; HLA, human leukocyte antigen; LN, liquid nitrogen; HES, hydroxyethyl starch; LC, leukocyte concentrate; DMSO, dimethyl sulfoxide.
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The second problem is that hematopoietic stem and progenitor cells can be affected by cryopreservation and thawing as usually performed, which reduces significantly the number of viable cells available for transplant. Considering this problem, Buckner *et al.* (15) observe that " … data suggest the feasibility of cryopreserving allogeneic marrow with the caveat that relatively large cell doses should be collected to compensate for the inevitable cell loss from marrow processing, cryopreservation and thawing." The methods described below overcome these obstacles.

MATERIALS AND METHODS

PCB Collection from the Delivered Placenta. Blood remaining in the delivered placenta and umbilical cord was retrieved by using protocols approved by the Institutional Review Boards of the New York Blood Center and Mount Sinai School of Medicine. The procedure, described previously (10), consists of inserting the 16-gauge needle of a standard 450-ml blood donor set containing CPD A anticoagulant (citrate/phosphate/dextrose/adenine) (Baxter Health Care, Deerfield, IL) into the umbilical vein of the delivered placenta and letting PCB drain by gravity into the blood bag. To facilitate collection and reduce the likelihood of contamination with maternal blood and secretions, the placenta is placed in a plastic-lined, absorbent cotton pad suspended from a specially constructed support frame. The 63 ml of CPD A used in the standard blood transfusion bag, calculated for 450 ml of blood, is reduced to 23 ml by draining 40 ml into a graduated cylinder just prior to collection. This volume of anticoagulant matches better the PCB volumes usually retrieved (<170 ml). Moreover, since CPD A is isotonic and has neutral pH, the ratio of anticoagulant to blood is not critical, as in the case of ACD (acid/citrate/dextrose).

Reduction of PCB Bulk: Preparation of Leukocyte Concentrates (LCs). After removal of aliquots for routine testing, 6% (wt/vol) hydroxyethyl starch (HES) (Hespan; DuPont) is added to the anticoagulated PCB to a final concentration of 1.2%—i.e., in a 1:5 volume ratio—to enhance its low red blood cell sedimentation rate (<1 mm/h without HES). A leukocyte-rich supernatant is then separated by centrifuging the PCB/HES mixture in the original collection blood bag (50 × g for 5 min at 10°C). The leukocyte-rich supernatant is expressed from the bag into a 150-ml Plasma Transfer bag (Baxter Health Care) and centrifuged (400 × g for 10 min) to sediment the cells. Surplus supernatant plasma is transferred into a second Plasma Transfer bag without severing the connecting tube. Finally, the sedimented leukocytes are resuspended in supernatant plasma to a total volume of 20 ml and are then designated as LC. These transfers can be done in a closed system, or the bags may be connected by the standard spike connectors with the usual precautions to prevent bacterial and fungal contamination.

Erythrocyte Sedimentation Rate, Blood Cell Count, and Cellular Viability. Sedimentation rates are measured in vertically held standard 5-mm (i.d.) glass tubes without correction for hematocrit. Erythrocyte, platelet, leukocyte, and differential leukocyte counts are performed with an automated cell counter (Technicon model H1). All counts are expressed as the total number of cells contained in the PCB unit. Viability is measured with a computer-driven Patimed microfluorimeter (Leica) by using DNA enhancement of ethidium bromide fluorescence as the index of cell death (16). One microliter of cell suspension, containing 5000–10,000 nucleated cells, is exposed to ethidium bromide at a final concentration of 1 μg/ml for 30 min prior to reading fluorescence emission in the 550- to 580-nm range. Viability is determined by least-squares linear regression: the coefficient of correlation (r^2) with known mixtures of heat-killed and live cells is ≥0.991. With appropriate magnification, the morphology of the fluorescent nu-

cleus of dead cells permits easy visual differentiation of mononuclear cells from segmented granulocytes.

Culture and Counting of Hematopoietic Precursors. Base culture medium, made locally in Iscove's modified Dulbecco's Medium (IMDM; GIBCO), contains 0.8% (wt/vol) methylcellulose, 30% (vol/vol) heat-inactivated fetal bovine serum (HyClone), 1% (vol/vol) antibiotic/antimycotic solution (GIBCO/BRL), and 7.5 × 10^{-5} M 2-mercaptoethanol. PCB is added in volume ratios of 1:250 or 1:500 into 2.5-ml base culture tubes, each of which also receives the following recombinant human growth factors: 50 ng of erythropoietin, 25 ng of granulocyte/macrophage colony-stimulating factor, 250 ng of stem cell factor (all provided by Amgen Biologicals), 250 ng of granulocyte colony-stimulating factor (gift from K. Kaushanski, University of Washington School of Medicine, Seattle), and 25 units of interleukin 3 (Genetics Institute, Cambridge, MA). Duplicate 1-ml samples from each tube are cultured in Petri dishes for 2 weeks at 37°C in a humidified, 5% O_2/5% CO_2/90% air controlled atmosphere. Erythroid bursts and granulocyte/macrophage and mixed-cell colonies are identified by microscopic examination and counted separately in the same dish, but data are presented as the total number of colonies in the PBC unit.

Cryopreservation. Dimethyl sulfoxide (DMSO) (Cryoserv, Research Industries, Salt Lake City) is used at a final concentration of 10% (vol/vol). The required volume of sterile, chilled DMSO solution is added to the blood bag over the course of 15 min by using a syringe pump and an orbital mixer to assure smooth but vigorous mixing. In these experiments, whole PCB units were mixed with 20% (vol/vol) DMSO in isotonic saline, and LCs were mixed with either 20% DMSO in saline or 50% DMSO in 5% (wt/vol) Dextran 40 (M_r ≈ 40,000) (Baxter Health Care). Final volumes for whole PCB units with DMSO became, thus, twice the volume of the originally collected PCB plus the anticoagulant, whereas the final volume of LCs with DMSO was a uniform 25 ml. Cryoprotectant and PCB or LC are kept cold with wet ice throughout the addition. When the concentration of DMSO reaches 10%, cell suspensions are transferred to Cryocyte freezing containers (Baxter Health Care), placed in aluminum canisters, and then deposited horizontally on a level surface inside a −80°C freezer (Revco, Asheville, NC). When their temperatures are below −50°C, the units are transferred to the liquid phase of a LN freezer for storage.

Thawing Units After Storage in LN. Units for experimental work are kept in LN for at least 48 h. To thaw the unit, the bag is lifted to the gas phase of LN for 15 min and then exposed to ambient air for 5 min to allow the plastic to regain some elasticity. The bag is then immersed in 37°C water for thawing as rapidly as possible, usually <2 min for a whole blood unit.

Removal of Hypertonic Cryoprotectant. Immediately after being thawed, each PCB unit is diluted with an equal volume of a solution containing 2.5% (wt/vol) human albumin (Cutter) and 5% (wt/vol) Dextran 40 (Baxter Health Care) in isotonic salt solution, with continuous mixing, and then centrifuged at 400 × g for 10 min. The supernatant is removed, and the sedimented cells are resuspended slowly in fresh albumin/dextran solution to a volume appropriate for infusion to patients or, in these experiments, to the volume originally collected.

Statistical Tests. Routines within SPSS (Statistical Package for the Social Sciences) were used for the estimates shown.

RESULTS

Erythrocyte Sedimentation Rates of PCB After the Addition of HES. The sedimentation rates of two aliquots from each of 12 PCB units were measured at 1 and 3 h. One aliquot was diluted with a 1:5 volume of normal saline, and 1 was diluted with HES. All 12 samples to which saline was added had

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Table 1. Leukocytes and hematopoietic progenitors in PCB units and their respective LCs

Cell type	Mean cell count/unit, $\times 10^{-6}$		LC/PCB, %
	PCB	LC	
Leukocytes ($n = 390$)	958 ± 29.0	869 ± 27.8	91
Neutrophils	451 ± 19.1	389 ± 17.9	86
Lymphocytes	261 ± 21.6	266 ± 24.9	102
Progenitors ($n = 58$)	1.08 ± 0.1	1.06 ± 0.1	98

Leukocytes and hematopoietic progenitor cells were counted in aliquots from PCB units before (PCB) and after subtracting red cell mass and concentrating leucocytes (LC). Data are presented as the mean total cell counts $\pm$ SEM for the complete volume of the PCB unit and the LC, respectively.

sedimentation rates of <1 mm at 1 h and <2 mm at 3 h, while aliquots with HES sedimented at rates of 7–10 mm/h for the first 3 h.

Leukocytes and Progenitor-Cell Concentrations in LC. Table 1 summarizes the numbers of leukocytes and progenitor cells in PCB units before and after erythrocyte separation by HES, centrifugation, and final resuspension to 20 ml (LC). There is a small but significant decrease in the average total leukocyte numbers and a smaller and nonsignificant loss of progenitor cell numbers. The numbers of lymphocytes in LCs are essentially identical to those in the original PCB collections, but there is a small loss of granulocytes. Thus, lymphocyte concentrations are proportionately higher in LCs than in their respective PCB units.

Addition of 50% DMSO as a Cryoprotectant. Twelve LCs were used to assess the possibility that concentrated DMSO might have deleterious effects on either the viability of leukocytes or the clonogenic activity of progenitor cells. For this analysis, each LC was divided into two aliquots; one was protected by adding the regular 20% DMSO solution, and the other was treated with 50% DMSO in 5% Dextran 40, both to a final DMSO concentration of 10%. (Preliminary experiments showed that the presence of dextran in a final concentration of 1% marginally improved the viability of leukocytes in PCB units or LCs by 3–10%.) The results of using each of the cryoprotectant solutions, after thawing with removal of the supernatants as described above, are shown in Table 2. None of the differences between the two concentrations was significant in two-tailed paired *t* tests.

Effect of Removing Cryoprotectant on the Recoveries of Viable Leukocytes and Hematopoietic Progenitor Cells from Frozen/Thawed PCB Suspensions. These measurements were made to compare the viability and clonogenic activity of PCB leukocytes tested immediately after thawing with either the conventional technique (1) or after removal of DMSO. Thir-

Table 2. Leukocytes and hematopoietic progenitors in 12 LC units before freezing and after cryoprotection with 20% or 50% DMSO

Cell type	Mean cell count/unit, $\times 10^{-6}$		
	Fresh	Thawed	
		20% DMSO	50% DMSO
Total leukocytes	992 ± 97.2	913 ± 111.0	937 ± 103.1
Viable leukocytes	952 ± 75.7	575 ± 69.6	603 ± 74.3
Neutrophils	523 ± 62.9	164 ± 21.5	149 ± 19.2
Lymphocytes	317 ± 45.8	279 ± 42.6	299 ± 44.1
Progenitors	1.08 ± 0.29	0.97 ± 0.29	1.03 ± 0.33

Total and viable (ethidium bromide-excluding) leukocytes were counted in aliquots from LC units before they were frozen in 10% (wt/vol) DMSO added from either 20% or 50% (wt/vol) solutions (Fresh) and after they were thawed. Data are presented as the mean total cell counts $\pm$ SEM for the complete volume of the LC unit.

Table 3. Leukocytes and hematopoietic progenitors in PCB units, before freezing and after thawing in the presence or absence of cryoprotectant

Cell type	Mean cell count/unit, $\times 10^{-6}$			P^*
	Fresh	Thawed		
		+ DMSO	− DMSO	
Total leukocytes	944 ± 73.1	883 ± 70.7	867 ± 78.4	NS
Viable leukocytes	895 ± 70.4	315 ± 40.2	543 ± 71.9	<0.0001
Neutrophils	478 ± 36.0	109 ± 21.5	146 ± 28.2	0.013
Lymphocytes	267 ± 45.8	156 ± 33.6	264 ± 47.4	<0.0001
Progenitors	1.10 ± 0.28	0.68 ± 0.29	1.29 ± 0.33	0.0004

Cells and hematopoietic colonies in PCBs ($n = 13$) were counted before being frozen in 10% DMSO (Fresh) and after being thawed. Cell numbers and viabilities were determined in thawed cell suspensions, either as just thawed, in the presence of DMSO (+ DMSO), or in aliquots from which supernatant DMSO was removed after a 1:2 dilution with a 10% (wt/vol) dextran solution of osmolarity ≈ 310 mosmol/liter (− DMSO). Data are presented as the mean total cell counts $\pm$ SEM for the complete volume of the PCB unit. NS, not significant.
*Paired *t* test, two-tailed, comparing the two postthaw counts.

teen PCB units were thawed and two aliquots were taken immediately from each. One of these aliquots was processed and tested without removing DMSO ("unwashed" aliquot), while the other was processed to eliminate DMSO ("washed") immediately after thawing. Samples from each aliquot were mixed with ethidium bromide (for viability testing) or with culture medium (for progenitor cell assays) as soon as the DMSO was removed from the "washed" aliquot (within 15 min of thawing). The results are summarized in Table 3.

In each case, the new procedure resulted in increased leukocyte viability, as determined by both direct cell counts and formation of hematopoietic cell colonies. Failure to remove cryoprotectant solution results in a 65% reduction in the viability of the leukocytes and nearly 40% in the viability of the hematopoietic progenitor cells, with respect to cells in fresh PCB units. Removal of DMSO reduced the mean loss of viable leukocytes to 39%, while losses of progenitor cells were undetectable in this experiment. To simulate more closely the fate of cryoprotected PCB after infusion into the recipient, cells were thawed and rapidly mixed with 20 volumes of autologous plasma. Rapid 20-fold dilution led to declines of 70–80% in the viability of unwashed cells but did not change the viability of washed cells (results not shown). Morphologic examination confirmed that a larger proportion of polymorphonuclear leukocytes than mononuclear cells were killed by DMSO-associated cell damage.

DISCUSSION

Favorable early results of HLA-matched, unrelated-donor PCB transplants for bone marrow reconstitution suggest that larger clinical trials may be warranted. Since the probability of finding an HLA-matched unit depends on the number of units available and since the number of units that can be stored in a given space is determined by the average unit volume, smaller PCB units are essential for PCB to become a practical source of tissue for unrelated transplantation.

Reducing the volume of the PCB units by eliminating red blood cell bulk and plasma offers pragmatic advantages of convenience, cost, and efficiency, compared with current alternatives, such as gradient separation of mononuclear cells or positive selection of CD34+ hematopoietic precursors. Therefore, we designed a closed-system procedure based on HES-induced rouleaux formation and mild centrifugation to separate a leukocyte-rich supernatant from sedimented red blood cells. The system is compatible with routine blood bank processing and concentrates almost all the leukocytes and

progenitor cells in PCB units (volumes up to 170 ml have been tested, which would have resulted in 350 ml of stored volume after addition of 20% DMSO) into a volume of 20 ml. To make the final volume as small as possible, we used 50% DMSO in the cryoprotectant solution instead of the traditional 20% DMSO. Cryopreservation with concentrated DMSO solution maintained the clonogenic function of PCB leukocyte suspensions as well as the 20% solution did, provided its addition to LCs was slow and even, with efficient mixing and in the cold. Final unit volume, including cryopreservative, was constant and amounted to only 25 ml. This smaller volume multiplies freezer capacity 3-fold when storing PCB units in conventional 250-ml cryobags and metal canisters and could result in a 10-fold increase with a specially designed storage system. Reducing the volume of PCB units also should help maintain their high cell viability, as the smaller volume allows them to be frozen more uniformly and thawed more rapidly. We have recently confirmed the capacity of volume-reduced units to reconstitute hematopoiesis: one of these units was transplanted on February 21, 1995, and engraftment followed promptly (nucleated cell count of 500 at day 16) with nucleated cells of only donor type in the blood and marrow of the patient.

The second issue addressed here is related to the "toxicity" of DMSO. Progenitor cell suspensions in cryoprotective media have been shown to have poor viability and clonogenic activity, even if tested quickly after thawing. This loss of viability is manifest *in vivo*, after allogeneic bone marrow transplantation (15), and *in vitro*, when thawed PCB cell suspensions, still in 10% DMSO, are diluted 1:200 or 1:400 in isotonic solutions for cell counting or in tissue culture medium for clonogenic assays (11). In our experiments, loss of viability was minimized by adding one volume of isotonic diluent with osmolarity $\approx$310 mosmol/liter to the cell suspension in 10% DMSO, which suggests that hyperosmolarity (17) (10% DMSO $\approx$ 1.25 M) and osmotic shock upon a brusque reduction of osmolality may be responsible. Similar observations were reported in 1978 by Goldman *et al.* (18), who showed that clonogenic activity declined rapidly in thawed bone marrow and peripheral blood cells suspended in 10% DMSO and that this decline could be largely prevented by a prompt 1:2 dilution in saline. These authors credited E. D. Thomas (Fred Hutchinson Cancer Research Center, Seattle) with suggesting the 1:2 postthaw dilution as a means of reducing granulocyte aggregation.

Dilution of the freshly thawed cryopreserved unit with an equal volume of isotonic solution also allows the sample to be thawed and prepared for infusion in the laboratory under controlled conditions rather than at the bedside. Removal of the cryoprotective medium can be performed there, without harm to the units: clonogenic activity and viability remain essentially unchanged, even if removal of diluted DMSO is delayed for several hours. Furthermore, removing DMSO eliminates its deleterious effects on the transplant recipient (19, 20).

Conceivably, the same procedure could be applied to cryopreserved bone marrow and peripheral blood stem and progenitor cells to improve viability and allow more time between thawing a unit and infusing it. During that time, there might be an opportunity, for example, to purge or remove contam-

inating malignant cells or to introduce chemical or genetic labels into cells before infusion.

These improvements make frozen PCB a more effective source of stem cells for unrelated marrow reconstitution in the treatment of inherited disease and hematologic malignancy and, potentially, for recombinant DNA-based gene therapy.

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